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Transition-state analogues as inhibitors for GABA-aminotransferase

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Summary — Our previous calculations on the reaction catalysed by GABA- aminotransferase (GABA-T) have been utilized in this work in order to synthesize a series of reversible inhibitors of this enzyme. The synthesized transition-state analogues and their precursors inhibited GABA-T competitively in both the holoenzyme and apoenzyme at 10^{-3} and 10^{-5} M, respectively. In the case of the holoenzyme, the transition-state analogue series (the conformationally restricted series) gave a significant increase in inhibition values over the open (less conformationally restricted) series.

Résumé — Inhibiteurs de GABA-transaminase analogues de l'état de transition. *Dans cette étude, nous rapportons la synthèse d'une série d'inhibiteurs réversibles de GABA-transaminase (GABA-T). Nous y utilisons les résultats de calculs antérieurs du mécanisme de la réaction catalysée par GABA-T. Les structures d'état de transition et leurs précurseurs inhibent GABA-T de façon compétitive, aux niveaux de l'holoenzyme et de l'apoenzyme. Dans le cas de l'holoenzyme, les structures d'états de transition (à conformation restreinte) sont de bien meilleurs inhibiteurs que les précurseurs, à conformation moins restreinte.*

GABA-aminotransferase / transition-state analogues / biochemical evaluation / enzyme inhibitors

Introduction

This work is an approach to the synthesis of a new class of inhibitors for gamma-aminobutyrate aminotransferase (EC 2.6.1.19, GABA-T). The significance of the biochemical pathway of GABA-T is the conversion of a major inhibitory neurotransmitter, gamma-aminobutyrate [1–4], into succinic semi-aldehyde in the mammalian central nervous system [5]. For some years GABA-T has been one of the most pharmacologically important target enzymes, especially since the discovery that inhibitors of this enzyme provide potential treatment for neuropsychiatric disorders, *eg* epilepsy [6], Parkinsonism [7], schizophrenia [8], tardic dyskinesia [9], and Huntington's disease [10, 11]. Indeed, over the last 2 decades a vast number of classes of inhibitors have been developed and studied [12]. Some of these inhibited GABA-T and produced anticonvulsant activity, but their action is generally not specific [13]. Recently, several irreversible (suicide) inhibitors of GABA-T have been developed and tested clinically for epilepsy [14], but there are as yet no potent and specific reversible inhibitors of the enzyme, although they are expected to be less toxic [15] and preferable for therapeutic use. We therefore present the synthesis and biochemical evaluation of a new class of inhibitors, transition-state analogues, as an alternative method for developing reversible inhibitors of GABA-T.

The design of transition-state structure

The use of transition-state analogues [16] is based on the fact that enzymes exert a substantial part of their catalytic activity by binding preferentially to the transition state of the reaction (fig 1), thus lowering the enthalpy of activation relative to that of the uncatalysed reaction. Stable molecules that mimic the geometry of the transition-state structure will therefore bind more tightly to the enzyme than substrate analogues, and should be powerful inhibitors of the enzyme. In an earlier study [17] we used the semi-empirical molecular-orbital (MO) method MINDO/3 to calculate the transition-state structure and energy for a model reaction representing the important features of the reaction catalysed by GABA-T. Recently [18], we extended our earlier work by using semi-empirical MO calculations to elucidate the role of the pyridoxal ring and its functional groups in the stabilization of the transition state.

These molecular-orbital calculations have provided us with a detailed picture of the stereochemistry of the

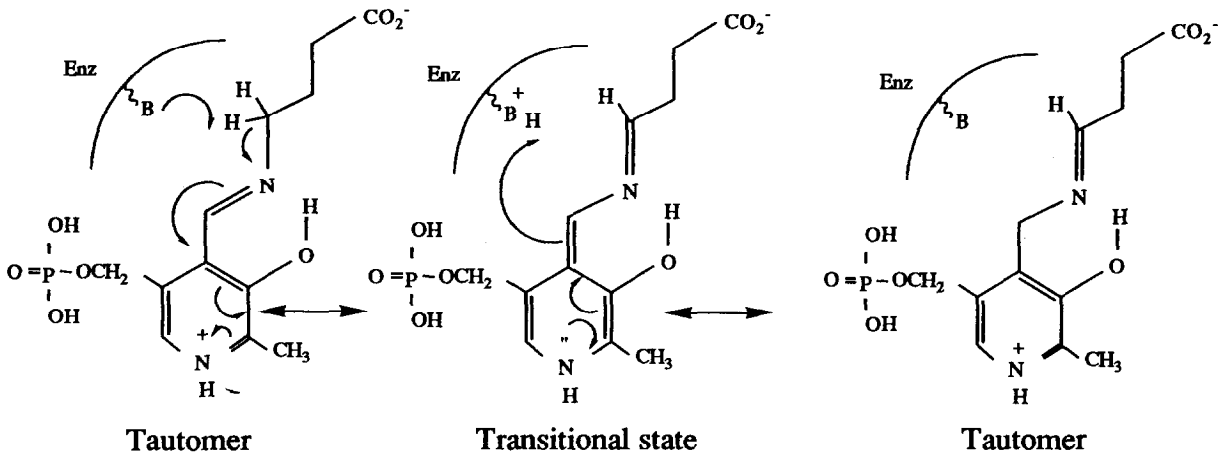

Fig 1. The transition state during the 1,3-prototropic shift in the GABA-T active site.

transition-state structure, illustrated in figure 2a. Although there is considerable flexibility in the placement of the carboxylate and phosphate sidechains in this structure, the calculations do define the mutual alignment of the co-enzyme and substrate in the active site, which is vital for catalysis to proceed. The calculated transition-state structure also suggests that structures which keep the Schiff-base nitrogen roughly in the pyridine plane may be useful.

The long partial bonds and abnormal hybridization of transition-state structures preclude precise correspondence between the functional groups of an analogue and those of the transition state, although a choice of suitable analogues can usually be made by superimposing their structures on that of the transition state using interactive graphics routines to manipulate either molecule. We automate this process by running a stepwise minimization of the extent of misfit between the 2 molecules. Analogues which optimally match the transition-state structure are then chosen for synthesis.

Chemistry

Oxazine compounds were suggested for the proposed analogues. The oxazine ring structure (fig 2b) fits ideally with the calculated transition state (fig 2a), and at the same time maintains all the heteroatoms at the correct positions, almost coplanar with the rest of the molecule. All the proposed compounds in figure 3 were synthesized in 2 major steps.

Series **I** was prepared by condensation of the aldehyde with the corresponding amino carboxylic acid in dry

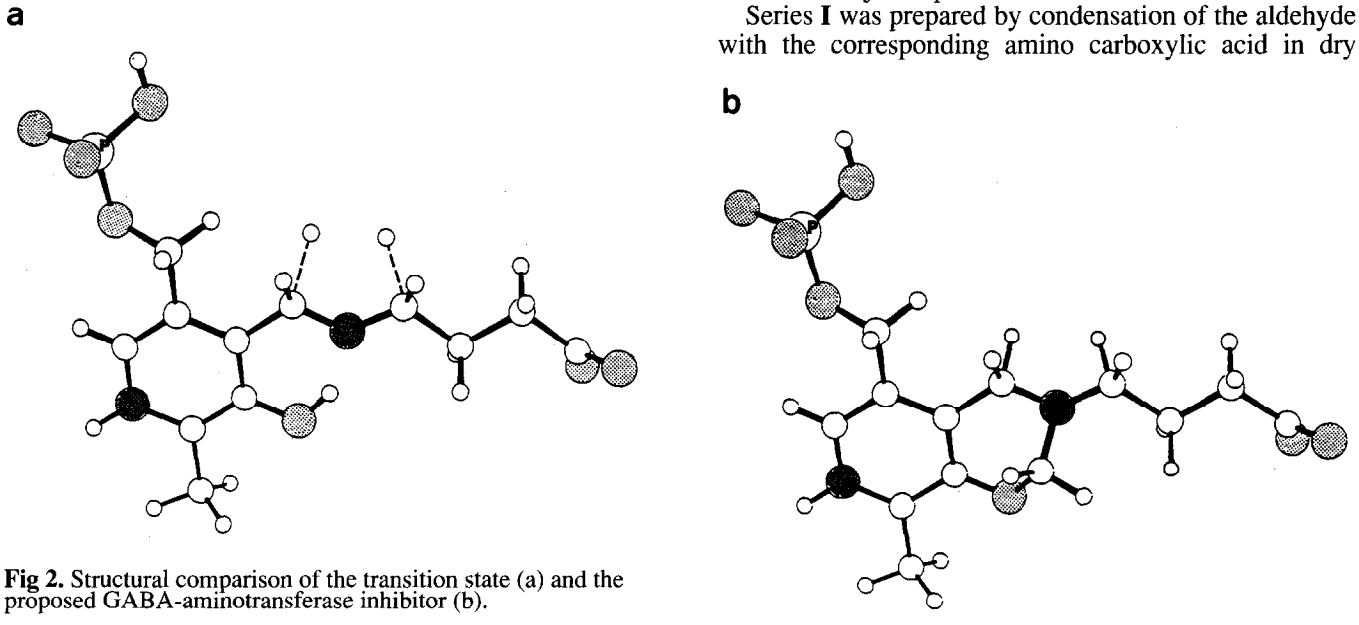

Fig 2. Structural comparison of the transition state (a) and the proposed GABA-aminotransferase inhibitor (b).

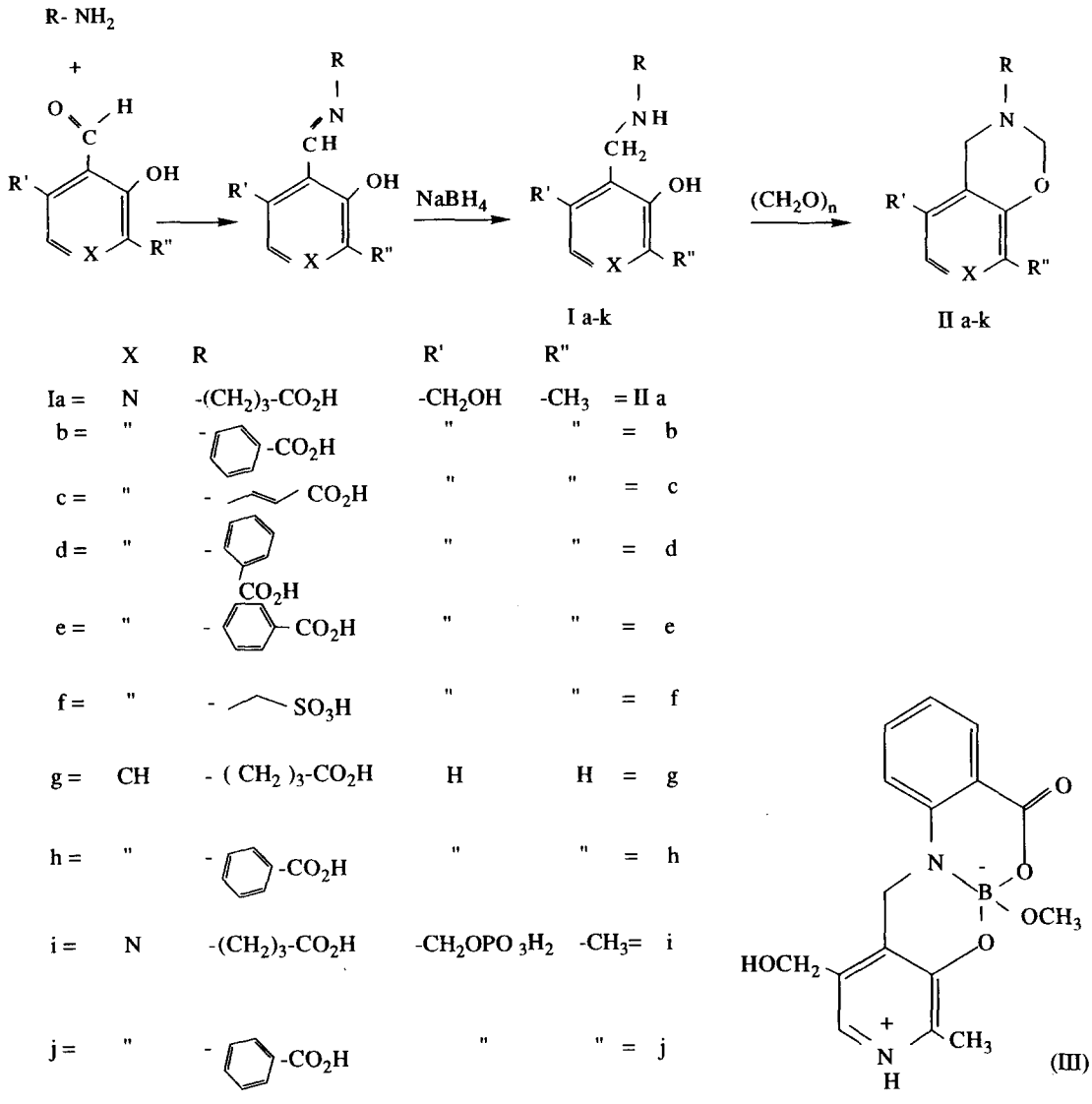

Fig 3. Synthesis of oxazine derivatives (**IIa–k**).

methanol, and subsequent treatment with sodium borohydride afforded the precursors (**Ia–c, Ie–j**) in good yield. Compounds **Ii** [19] and **Ij** [20] had been previously prepared, but no structural characterization was carried out. Compound **Id** had to be reduced with platinum oxide in the presence of hydrogen at room temperature. The reduction of the imine of compound **Id** with sodium borohydride under the reaction conditions described in **Ia** gave the tetra-cyclic benzoxazaborinone (**III**) in figure 3, rather than the desired secondary amine (**Id**). An account of the chemistry and crystal structure of the novel boron compound (**III**) has been published in an earlier paper [21]. Reaction of the secondary amines (**Ia–j**) with *para*-formaldehyde *via* Mannich cyclization reaction afforded the oxazines (**IIa–j**) in good yield.

Biochemical results and discussion

The transition-state analogues (**IIa–j**) and their precursors (**Ia–j**) were tested against the activity of pig brain GABA-T (under physiological conditions) and PLP-free GABA-T. When the PLP-free enzyme was used it was pre-incubated with the inhibitor for 30 min, and PLP was

then added to the apoenzyme during the assay to reach the normal level of PLP present in the holoenzyme. This effectively gives reconstituted PLP-bound GABA-T. This method allows the inhibitor to enter the active site if possible, and its displacement by the co-enzyme and GABA to be measured. It was clear from the biochemical studies that all the compounds in table I inhibited GABA-T competitively with respect to GABA in both the holoenzyme and apoenzyme. The conformationally restricted series (**II**) gave a significant increase in inhibition values over the open (less conformationally restricted) series (**I**) with the PLP-bound enzyme. The presence of the phosphate group in **Ij**, **IIi** and **IIj** enhanced inhibitory activity by 62, 49 and 44%, respectively, in comparison with the non-phosphorylated analogues, **Ib**, **IIa** and **IIb**, respectively. Interestingly, the phosphorylated compound **Ii** did not show a remarkable increase in activity over the non-phosphorylated analogue **Ia**. This could be due to the introduction of the bulky phosphate group, which may have distorted the molecule to an undesirable conformation. This is supported by results from the biochemical data: firstly, the partially restricted aromatic analogue **Ij** is not affected by the introduction of the phosphate group and shows the expected increase in activity. Secondly, the introduction of the phosphate group to the conformationally restricted analogue, **IIi**, does not alter the expected activity in comparison with the non-phosphorylated, conformationally restricted analogue **IIa**. Compound **Ij** was the only inhibitor in series **I** with an inhibition value equal to that of the corresponding restricted conformation analogue, **IIj**.

When series **I** and **II** were tested using PLP-free enzyme, a 100-fold enhancement of inhibitory activity was observed for all series **I** and **II** compounds when compared with the results using the co-enzyme-bound enzyme (table I).

The potency of compound **IIj** as a GABA-T inhibitor (the most active compound in the series) was determined from the following equation:

$$K_i = \frac{IC_{50}}{\frac{C}{1 + K_m}} \quad (1)$$

where C is the concentration of GABA.

The inhibitor **IIj**-enzyme dissociation constant (Ki) was calculated for both the holoenzyme and apoenzyme and gave 2 values of K_i, 4.6×10^{-5} M and 5.9×10^{-7} M, respectively. The low K_i value of **IIj** reflects strong affinity for the catalytic binding site.

Conclusion

In view of the biochemical results in table I, the series **II** (restricted) analogues were generally, under these physiological conditions, the better potential inhibitors and were much better at displacing the endogenous PLP and GABA from the active site. This is supported by our earlier work on X-ray crystal structures and conformations [22].

Table I. Activities of **Ia–j** and **IIa–j** as inhibitors of GABA-T.

Secondary amines	*% Inhibition*		*Oxazines*	*% Inhibition*	
	PLP-bound (10^{-3} *M*)	*PLP-free* (10^{-5} *M*)		*PLP-bound* (10^{-3} *M*)	*PLP-free* (10^{-5} *M*)
Ia	27	25	**IIa**	47	33
b	38	41	**b**	56	51
c	32	21	**c**	52	16
d	22	< 10	**d**	48	< 10
e	28	31	**e**	57	35
f	18	18	**f**	12	27
g	< 10	16	**g**	< 10	10
h	20	14	**h**	49	16
i	36*	33	**i**	97	88
j	100	99	**j**	100	98

*Lit 30% [14]

In the preceding study we found that series **II** fitted the calculated transition state better, and both the entropy and enthalpy contributions of this series to binding were more favourable than those of series **I**.

When series **I** and **II** were assayed against the PLP-free enzyme, both series inhibited the enzyme at a 10^{-5} molar level. In these conditions, series **II** was the more active by a small but significant margin.

It is clear from the present study that the transition-state analogues have produced considerable inhibitory activity against GABA-T. However, the potency of this species still depends on the presence or absence of PLP in the active site of the enzyme.

Experimental protocols

^{1}H NMR spectra were obtained with a Perkin–Elmer R12 spectrometer at 60 MHz and a Bruker AM 300WB multinuclear magnetic resonance spectrometer at 300 MHz. Chemical shifts (δ) were measured in ppm from internal $SiMe_4$. All spectra were consistent with the assigned structure. Mass spectra were determined on a Jeol JMS-DX300 double-focussing spectrometer. Melting points were determined on a Mettler FP21 hot-stage microscope with a Mettler FP2 recorder, and are uncorrected. Elemental analyses were carried out by the Australian Microanalytical Service (Amdel), Melbourne. Analytical figures were all within ± 0.4% of theoretical values.

4-[[[3-Hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridyl]methyl]-amino]butanoic acid ***Ia***

A solution of pyridoxal, free base (2.03 g, 10 mM) in dry methanol (35 ml) was added dropwise to a solution of potassium salt of γ-butyric acid (1.41 g, 10 mM) in dry methanol (30 ml). After 30 min continuous stirring, the yellow solution obtained was treated with sodium borohydride (0.38 g, 10 mM) at 5°C (added in portions). The reaction mixture was filtered, acidified with glacial acetic acid to pH 5, and then concentrated *in vacuo*. The residue obtained was dissolved in distilled water (5 ml) and transferred onto a column (2.8 x 50 cm) containing Dowex 50 WX (H^+), (20/50) ion exchange resin. The compound was washed firstly with distilled water (1000 ml) and then eluted with 1 M ammonium hydroxide solution. Aliquots of 5 ml each were checked regularly by TLC (water/acetone/*t*-amyl alcohol/glacial acetic acid, 20/35/40/5 silica gel). The aliquots containing the product (R_f 0.21) were combined, freeze-dried and the powder obtained further dried over phosphorus pentoxide *in vacuo*. This gave a 72% white powder (from ethanolic and ether), mp = 218–222°C, ^{1}H NMR δ (1% TMS in $CDCl_3$-C_2D_6O, 2/1) 1.7–2.1, m, 2H; 2.2–2.6, m, 2H; 2.5, s, 3H; 2.8–3.1, m, 2H; 4.2, s, 2H; 4.6, s, 2H; 7.7, s, ^{1}H. m/Z (70 eV) 236 (M^+ –18, 49%), 151 (61), 150 (30), 138 (63), 135 (13), 123 (82), 122 (15), 106 (14), 99 (22), 98 (27), 94 (34), 86 (100), 81 (10). Anal $C_{12}H_{18}N_2O_4$ (C, H, N). The preparation and work-up procedures of compounds **Ib**, **c**, **e**, **f**, **g**, and **h**, are treated as in **Ia**.

3-[[[3-Hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]methyl]-amino]benzoic acid ***Ib***

82% yield, white needles (from aqueous ethanol), mp = 209–210°C, R_f 0.88, ^{1}H NMR δ (D_2O, ext ref TMS in $CDCl_3$) 2.6, s, 3H; 4.7, s, 2H; 4.8, s, 2H; 7.3–7.5, m, 3H; 7.7, bs, 1H; 8.1, s, 1H. m/Z (70 eV) 288 (M+, 5%), 138 (9%), 137 (100), 120 (37), 92 (41), 91 (6), 66 (5), 65 (23), 64 (50). Anal $C_{15}H_{16}N_2O_4$ (C, H, N).

4-[[[3-Hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridyl]methyl]-amino]crotonic acid ***Ic***

Pale yellow powder (from ethanol-ether), 70% yield, R_f 0.21, ^{1}H NMR δ (D_2O, ext ref 1% TMS in $CDCl_3$) 2.3, s, 3H; 4.0, dd, J = 6.7 and 1.6 Hz; 4.1, s, 2H; 4.4, s, 2H; 5.8, dd, 1H, J = 15.3 and 1.6 Hz; 6.2–6.6, m, 1; 7.8, s, 1H. m/Z (70 eV) 234 (M+ –18, 13%), 216 (11), 169 (54), 151 (70), 150 (22), 137 (12), 136 (13), 123 (20), 122 (28), 120 (12), 107 (17), 106 (62), 95 (12), 94 (100), 92 (10), 82 (17), 81 (22), 80 (14), 67 (12), 67 (12). Anal $C_{12}H_{16}N_2O_4$ (C, H, N).

2-[[[3-Hydroxy-2-methyl-5-(hydroxymethyl)-2-methyl-4-pyridyl]-methyl]amino]benzoic acid ***Id***

A solution of pyridoxal, free base (2.03 g, 10 mM) in dry methanol (35 ml) was added dropwise to a solution of *o*-amino-benzoic acid (1.37 g, 10 mM) in dry methanol (30 ml). After 30 min continuous stirring, the yellowish-orange solution was reduced with platinum oxide and hydrogen at room temperature and atm pressure. The clear solution was filtered to remove the catalyst and concentrated. The solid product was crystallized from ethanol. This gave an 82% yield of colourless prisms, mp = 202–203°C, R_f 0.59, ^{1}H NMR (Bruker 300) δ (DMSO-d_6) 2.4, s, 3H, 4.4, s, 2H; 4.5, s, 2H; 6.6, t, 1H, J = 7.5 Hz; 6.9, d, 1H, J = 8.4 Hz; 7.4, ddd, 1h, J = 8.4, 8.5, 1.7 Hz; 7.8, dd, 1H, J = 8.0 and 1.8 Hz; 8.0, s, 1H. m/Z (70 eV) 288 (M^+, 14%), 138 (6.5), 137 (73), 120 (13), 119 (100), 93 (7), 92 (59), 91 (14), 65 (20), 64 (13), 63 (10). Anal $C_{15}H_{18}N_2O_4$ (C, H, N).

4-[[[3-Hydroxy-2-methyl-5-(hydroxymethyl)-2-methyl-4-pyridyl]-methyl]amino]benzoic acid ***Ie***

Fine pale-yellow prisms (from ethanol-diethyl ether), 60% yield, mp = 228–230°C, R_f 0.61, ^{1}H NMR δ (ethanol-d_6 and ? % TMS in $CDCl_3$, 4/1) 2.7, s, 3H; 4.7, s, 2H; 4.8, s, 2H; 6.9–7.1, m, 2H; 7.7–7.9, m, 2H; 8.1, s, ^{1}H. m/Z (70 eV) no molecular ion, 138 (M^+ –150, 9%), 137 (10), 121 (8), 120 (100), 93 (9), 92 (40), 74 (5), 66 (6), 65 (33), 64 (5). Anal $C_{15}H_{18}N_2O_4$ (C, H, N).

2-[[[3-Hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridyl]methyl]-amino]ethane sulphonic acid ***If***

Colourless prisms (from DMSO and water), 80% yield, mp = 215–216°C, R_f 0.17, ^{1}H NMR δ (0.5% DSS in D_2O) 2.5, s, 3H; 2.9–3.1, m, 4H, 4.6, s, 2H; 7.8, s, 1H. m/Z (70 eV) 194 (M^+ –H_2SO_3, 36%), 180 (5), 122 (8), 121 (12), 120 (59), 106 (7), 94 (15), 93 (16), 92 (16), 91 (5). Anal $C_{10}H_{16}N_2O_5S_5H_2O$ (C, H, N).

4-[[[2-Hydroxyphenyl]methyl]amino]butanoic acid ***Ig***

Colourless prisms (from water), 91% yield, mp = 184–185°C. ^{1}H NMR δ (1% DSS in D_2O) 1.9–2.2, m, 2H; 2.3–2.7, m, 2H; 3.0–3.3, m, 2H; 3.6, s, 2H; 6.3–6.6, m, 2H; 6.7–7.1, m, 2H. m/Z (70 eV) 209 (M^+, 17%), 191 (27), 136 (34), 135 (5), 122 (12), 121 (7), 108 (10), 107 (100), 106 (9), 84 (10), 79 (7), 78 (14), 77 (15). Anal $C_{11}H_{15}NO_3$ (C, H, N).

3-[[[2-Hydroxyphenyl]methyl]amino]benzoic acid ***Ih***

White plates (from ethanol-water), 94% yield, mp = 143–144°C (lit [23] 145–146°C) ^{1}H NMR δ (1% DSS in DMSO-d_6) 4.2, s, 2H; 6.5–7.5, m, 8H. m/Z (70 eV) 243 (M^+, 9%), 138 (9), 137 (100), 120 (29), 107 (7), 92 (30), 65 (18), 60 (41). Anal $C_{14}H_{13}NO_3$ (C, H, N).

4-[[[3-Hydroxy-2-methyl(5-[(phosphonoxy)methyl]-4-pyridinyl]-methyl]amino]butanoic acid ***Ii***

Pyridoxal-5-phosphate 2.65 g; 10 mM) was dissolved in dry methanol (40 µl) containing KOH (1.12 g, 20 mM). To the resulting yellow solution, a mixture of gamma-amino-butyric acid

(1.03 g, 10 mM) in dry methanol (40 µl) containing KOH (0.56 g, 10 mM) was added dropwise. After the addition, the reaction mixture was stirred for an additional 30 min, then sodium borohydride (0.38 g, 10 mM) was added portion-wise at 5°C. The reaction mixture was stirred for a further 10 min, filtered off, acidified with glacial acetic acid to pH 6, and concentrated *in vacuo*. The solid was dissolved in water (5 µl) and chromatographed on a column of DEAE-cellulose (60 x 3 cm). The compound was eluted with a solution of 0.3 M acetic acid and 0.3 M pyridine. Fractions of 10 µl were collected and checked by thin-layer chromatography (acetone/*t*-amylacohol/water/diethylamine, 3/4/2/1/silica gel). The correct fraction (R_f 0.51) was combined and concentrated to dryness *in vacuo*, and gave a 75% yield of white powder (from ammonia-saturated ethanol). ^{1}H NMR δ (D_2O, ext ref 1% TMS in $CDCl_3$) 2.1–2.4, m, 2H; 2.5–2.7, m, 2H; 2.7, s, 3H; 3.2–3.6, m, 2H; 4.6, s, 2H; 5.1, d, 2H, J = 7.3 Hz; 7.9, s, 1H. m/Z (70eV) no parent molecular ion; 236 ($M^+ - H_3PO_4$, 27%), 218 (40), 164 (12), 163 (100), k 151 (27), 150 (18), 149 (42), 138 (30), 136 (32), 135 (38), 123 (32), 107 (41), 106 (16), 98 (14), 94 (13), 86 (47), 84 (11), 66 (32), 65 (11).

3-[[[3-Hydroxy-2-methyl-5-[(phosphonyooxy)methyl]pyridyl]-methyl]-amino]benzoic acid ***Ij***

The preparation and work-up procedures for compound **Ij** were treated as in **Ii**. 84% yield white needles (from water-ethanol), mp = 208–210°C, R_f 0.4, ^{1}H NMR δ (DMSO-d_6, ext ref TMS in DMSO-d_6, Bruker AM 300 WB) 2.4, s, 3H; 4.3, s, 2H; 5.0, d, 6 Hz; 6.9–7.0, m, 1H; 7.3–7.4, m, 2H; 7.3, s, 1H; 8.1, s, 1H. m/Z (70 eV) no parent molecular ion. (Found: M^+ 98, 270.102, $C_{15}H_{14}N_2O_3$ requires M_+ –98, 280.101), 271 (14%), 270 (76), 269 (55), 253 (17), 241 (10), 225 (11), 224 (6), 149 (9), 148 (6), 138 (8), 137 (100), 134 (6), 121 (99), 120 (38), 106 (12), 94 (9), 93 (7), 92 (41), 91 (7), 80 (7), 77 (7), 66 (100), 65 (38).

4-(5-Hydroxymethyl-8-methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)butanoic acid ***IIa***

To a solution of *para*-formaldehyde (0.3 g, 10 mM) in dry methanol (50 ml), potassium hydroxide (20 mg) was added to dissolve the *para*-formaldehyde, 4-(3-hydroxy-5-hydroxymethyl-2-methyl-pyridin-4-yl-methyl)amino-*n*-butyric acid (**Ia**) was added and the reaction mixture refluxed. After 2 h the reaction mixtures was cooled, then concentrated *in vacuo*. The resultant residue was dissolved in a mixture of chloroform/methanol (3/1) and dried over $MgSO_4$. Concentration of the dried extracts gave **IIa** as a pale-yellow powder in 82% yield. ^{1}H NMR δ (1% TMS in $CDCl_3$-ethanol-d_6, 1/1) 1.9,s, 3H; 2.3–2.4, m, 4H; 2.6–2.8, 2H; 4.0, s, 2H; 4.5, s, 2H; 4.8, s, 2H; 7.8, s, 1H. m/Z (70 eV) 267 (M^+ +1, 12%), 266 (67), 249 (12), 248 (63), 194 (11), 193 (100), 179 (33), 163 (14), 152 (11), 151 (17), 150 (16), 149 (12), 123 (14), 122 (17), 116 (270), 106 (44), 98 (20), 94 (60). Anal $C_{13}H_{18}N_2O_4$ (C, H, N).

The preparation and work-up procedures of compounds **IIb-IIh** were treated as in IIa.

m-(5-Hydroxymethyl-8-methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)benzoic acid ***IIb***

White plates (from methanol), 94% yield, mp = 136–138°C, ^{1}H NMR δ (ethanol-d_6-1% TMS $CDCl_3$, 1/1) 2.6, s, 3H; 4.7, s, 2H; 4.9, s, 2H; 5.7, s, 2H; 7.2–7.8, m, 4H; 8.2, s, 1H. m/Z (70 eV) 301 (M^+ +1, 7%), 300 (36), 282 (19), 269 (8), 153 (9), 152 (7), 151 (10), 150(19), 149 (100), 148 (22), 137 (6), 122 (5), 121 (11), 120 (5), 106 (14), 94 (20), 81 (65), 77 (9), 65 (14). Anal $C_{16}H_{16}N_2O_4$ (C, H, N).

4-(5-Hydroxymethyl-8-methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)crotonic acid ***IIc***

Pale-yellow powder (from ethanol-diethyl ether), 65% yield. ^{1}H NMR δ (C_2D_6O-1% TMS in $CDCl_3$, 1/1) 2.4, s, 3H; 4.1, dd, 2H, J = 6.7 and 1.6 Hz; 4.4, s, 2H; 4.8, s, 2H; 5.7, s, 2H; 5.9, dd, 1H, J = 15.3 and 1.6 Hz; 6.3–6.7, m, 1H; 8.2, s, 1H. m/Z (70 eV) 264 (M^+, 12%), 246 (11), 204 (12), 169 (38), 163 (12), 162 (10), 152 (15), 151 (100), 150 (36), 138 (12), 136 (15), 133 (11), 123 (35), 122 (32), 108 (10), 107 (19), 106 (67), T 95 (12), 94 (86), 86 (12), 82 (18), 81 (21), 80 (16). Anal $C_{13}H_{16}N_2O_4$ (C, H, N).

o-(5-Hydroxymethyl-8-methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)benzoic acid ***IId***

White prisms (from ethanol), 74% yield, mp = 212–215°C, ^{1}H NMR δ (CD_3OD-1% TMS in $CDCl_3$, 1/1) 2.5, s, 3H; 4.5, s, 3H; 4.7, s, 2H; 5.3, s, 2H; 6.4–6.9, m, 2H; 7.3–7.7, m, 2H; 8.0, s, 1H. m/Z (70 eV) 300 (M+, 9%), 151 (9), 150 (5), 149 (7), 138 (9), 137 (99), 121 (10), 120 (100), 106 (6), 94 (9), 93 (6), 92 (34). Anal $C_{16}H_{16}N_2O_4$ (C, H, N).

p-(5-Hydroxymethyl-8-methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)benzoic acid ***IIe***

White prisms (from ethanol-diethyl ether), 58% yield, mp = 205–206°C, ^{1}H NMR δ (CD_3OD-1% TMS $CDCl_3$, 1/1) 2.4, s, 3H; 4.6, s, 2H; 4.8, s, 2H; 5.8, s, 2H; 6.6–6.7, m, 2H; 7.4–7.5, m, 2H; 8.1, s, 1H. m/Z (70 eV) 301 (M^+ +1, 9%), 300 (50), 283 (8), 282 (34), 269 (11), 153 (8), 152 (8), 151 (10), 150 (19), 149 (100), 148 (17), 102 (12), 123 (6), 122 (15). Anal $C_{16}H_{16}N_2O_4$ (C, H, N).

2-(5-Hydroxymethyl-8-methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)ethane-sulphonic acid ***IIf***

Colourless needles (from dimethylsulphoxide), 85% yield, mp = 234–235°C (dec), ^{1}H NMR δ (1% DSS in DMSO-d_6, Jeol FX 90A at 90 MHz), 2.5, s, 3H; 2.9–3.1, m, 2H; 3.3–3.5, m, 2H; 4.2, s, 2H; 4.6, s, 2H; 5.0, s, 2H; 7.9, s, 1H. m/Z (70 eV) 288 (M^+, 13%), 168 (19), 154 (11), 153 (100), 152 (43), 151 (46), 150 (25), 139 (13), 128 (23), 136 (26), 135 (46), 124 (75), 123 (23), 122 (26), 110 (26), 108 (17), 107 (25), 106 (29), 94 (60), 82 (15), 81 (21), 80 (23). Anal $C_{11}H_{16}N_2O_5S$ (C, H, N).

4-(3,4-Dihydro-1,3-benzoxazin-3-yl)butanoic acid ***IIg***

White plates (from methanol-water), 86% yield, mp = 228–230°C, ^{1}H NMR δ (1% DSS in DMSO-d_6) 1.5–1.7, m, 2H; 1.8–2.0, m, 2H; 2.2–2.5, m, 2H; 3.4, s, 2H; 4.7, s, 2H; 6.1–6.4, m 2H; 6.6–6.9, m, 2H. m/Z (70 eV) 221 (M^+, 2%), 209 (16), 191 (25), 136 (33), 135 (7), 134 (5), 121 (6), 108 (10), 107 (100), 106 (15), 84 (10), 79 (7), 78 (18), 77 (4). Anal $C_{12}H_{15}NO_3$ (C, H, N).

m-(3,4-Dihydro-1,3-benzoxazin-3-yl)benzoic acid ***IIh***

White plates (from ethanol-water), 91% yield, mp = 151–152°C, ^{1}H NMR δ (1% DSS in DMSO-d_6) 4.5, s, 2H; 5.4, s, 2H; 6.6–6.7, m, 8H. m/Z (70 eV) 256 (M^+ +1, 8%), 255 (49), 254 (12), 150 (10), 149 (100), 148 (29), 121 (9), 78 (13), 77 (11), 65 (9). Anal $C_{15}H_{13}NO_3$ (C, H, N).

4-(8-Methyl-5-phosphonooxy)methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)butanoic acid ***IIi***

This compound was synthesized by the same method as compound **IIa** and obtained as a white powder (from ethanol-diethylether), 85% yield, ^{1}H NMR δ (1% TMS in $CDCl_3$-C_2D_6O, 1/1), 2.2, s, 3H; 2.2–2.5, m, 2H; 2.5–2.7, m, 2H; 3.4–3.6, m, 2H; 4.5, s, 2H; 4.8, s, 2H; 5.0, d, 2H, J = 7.3 Hz; 7.7, s, 1H. m/Z (70 eV) no parent molecular ion. 266 (M^+ –80, 10%), 248 (12), 236 (70), 220 (70), 204 (10), 193 (20), 177 (24), 164 (80), 163 (100), 151 T (45), 138 (90), 136 (95), 123 (98), 107 (47), 49 (99), 98 (96), 86 (98). Anal $C_{13}H_{19}N_2O_7P$ (C, H, N).

m-(8-Methyl-5-(phosphonooxy)methyl-3,4-dihydropyrido[4,3-e]-1,3-oxazin-3-yl)benzoic acid ***IIj***

This compound was synthesized by the same method as compound **IIa**, except that dimethylformamide was used as the

reaction solvent instead of methanol. White needles (from dimethylform-amidediethylether), 91% yield, mp = 218–222°C, ^{1}H NMR δ (DMSO-d_6, ext ref TMS in DMSO-d_6, Bruker AM 300WB) 2.2, s, 3H; 4.7, d, 2H, J = 6 Hz; 4.8, s, 2H; 5.5, s, 2H; 7.4–7.5, m, 3H; 7.8, s, 1H; 8.0, s, 1H. m/Z (70 eV) no parent molecular ion. (Found: M^+ –79, 300.113, $C_{16}H_{16}N_2O_4$ requires M^+ –79, 300.111); 300 (27%), 284 (26), 281 (20), 271 (16), 270 (84), 27 (18), 253 (35), 241 (12), 225 (22), 224 (12), 151 (20), 150 (29), 149 (100), 148 (33), 137 (70), 136 (14), 135 (26), 134 (13), 121 (22), 120 (23). Anal $C_{16}H_{17}N_2O_7P$ (C, H, N).

Biochemical method

Materials

GABA, α-ketogluturate, NAD and Tris were purchased from Sigma Chemical Co and the ammonium sulphate, KH_2PO_4, K_2HPO_4, TCA and Triton X-100 from Ajax Chemicals Pty Ltd. Succinic acid, ß-mercaptoethanol and toluene were from BDH (Chemicals Ltd, UK; Dowex 50W x 4 (H+) was obtained from Fluka, Switzerland, [1-^{14}C]GABA, specific activity 50.4 mCi/mmol, from DuPont NEN Products, USA, dithiothreitol from Boehringer Mannheim GmbH, Germany, PPO from Canberra Packard, and PLP from the Aldrich Chemical Co.

Enzyme preparation (pyridoxal phosphate-free): GABA-T was partially purified from pig brain using the method of Churchich and Moses [24]. A suspension of this partially purified enzyme was dialysed overnight against 250 ml of 100 mM potassium phosphate (pH 7.4) containing 1 mM ß-mercaptoethanol. GABA in Tris-HCl at pH 8.5 was added to a final concentration of 4.0 mM, followed by incubation at room temperature for 20 min. Approximately 1 600 µl of 1.0 mM KH_2PO_4 was added to bring the pH to 6.0 and incubated for 30 min at 4°C. The solution was then dialysed against 250 ml of 100 mM Tris-HCl (pH 8.5) containing 1 mM of ß-mercaptoethanol for at least 2 h. PLP-free GABA-T activity with no PLP added (base levels) was 250–300 dpm, whereas the activity after addition of 33.3 µM PLP (controls) was 1100–1500 dpm under the same assay conditions. (PLP-bound GABA-T controls were 1600–1700 dpm).

Enzyme assay

The radiochemical method used for GABA-T was based on that of Beart *et al* [25]. A 150 µl aliquot of the PLP-free, partially purified GABA-T was preincubated with 45 µl of inhibitor dissolved in 100 mM Tris-HCl at pH 8.5 (assay buffer) in a shaking water-bath for 30 min at 37°C. PLP was added to a level of 33.3 µM, followed by incubation for 30 min at 37°C. Finally, 1.17 mM GABA (containing 4.2 µM of a 50.4 mCi/mmol [1-^{14}C] GABA), and 100 µl of stock solution containing 440 µM α-ketogluturate, 200 µM succinic acid, 2.22 mM dithiothreitol, and 0.67 mM NAD^+ was added. The assay solutions were made up to a final volume of 450 µl with assay buffer and incubated for 30 min at 37°C. The reaction was stopped by addition of 50 µl 25% TCA. The tubes were centrifuged at 2000 rpm for 5 min using an MSE bench centrifuge. The supernatants were transferred to separate columns (1 x 5 cm of Dowex 50W x 4 (H^+) resin (100–200 mesh), and eluted with 4.5 ml of distilled water. A 1 ml sample of the elutant was added to 10 ml of toluene/triton X-100/PPO scintillation cocktail and counted for 5 min in a Packard Tri-Carb 460C scintillation counter. Assays were carried out in triplicate with controls and blanks.

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